Chemistry & Biology, Vol. 12, 131–140, January, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2004.12.005

Tailoring of Glycopeptide Scaffolds by the Acyltransferases from the Teicoplanin and A-40,926 Biosynthetic Operons

Ryan G. Kruger,[1] Wei Lu,[1] Markus Oberthür,[1] Junhua Tao,[3] Daniel Kahne,[1,2] and Christopher T. Walsh[1,*]

[1]Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
Boston, Massachusetts 02115

[2]Department of Chemistry and Chemical Biology
Harvard University
Cambridge, Massachusetts 02138

[3]Pfizer Incorporated
San Diego, California 92121

Summary

The teicoplanin acyltransferase (Atf) responsible for *N*-acylation of the glucosamine moiety to create the teicoplanin lipoglycopeptide scaffold has recently been identified. Here we use that enzyme (tAtf) and the cognate acyltransferase from the related A-40,926 biosynthetic cluster (aAtf) to evaluate specificity for glycopeptide scaffolds and for the acyl-CoA donor. In addition to acylation of 2-aminoglucosyl glycopeptide scaffolds with k_{cat} values of 400–2000 min^{-1}, both Atfs transfer acyl groups to regioisomeric 6-aminoglucosyl scaffolds and to glucosyl scaffolds at rates of 0.2–0.5 min^{-1} to create variant lipoglycopeptides. Using the teicoplanin glycosyltransferase tGtfA, tAtf, and GtfD, a glycosyltransferase from the vancomycin producer, it is possible to assemble a novel lipoglycopeptide with GlcNAc at β-OH-Tyr_6 and an N_6-acyl-glucosaminyl-vancosamine at $Phegly_4$. This study illustrates the utility of chemo- and regioselective acyltransferases and glycosyltransferases to create novel lipoglycopeptides.

Introduction

The family of glycopeptide antibiotics is composed of two subfamilies containing the clinically useful antibiotics vancomycin and teicoplanin, respectively (Figure 1A). Each has an oxidatively crosslinked heptapeptide scaffold constructed by nonribosomal peptide synthetase assembly lines [1, 2], with sugars added by dedicated glycosyltransferases (Gtfs) in post assembly line tailorings (Figure 1B). Vancomycin has the disaccharide L-vancosaminyl-1,2-D-glucose attached to the phenolic hydroxyl of phenylglycine$_4$ ($Phegly_4$) of the scaffold by the tandem actions of GtfE and GtfD [3, 4]. Teicoplanin is glycosylated with glucosamine on $Phegly_4$ by tGtfB and is then *N*-acylated, capping the sugar against further elongation. A separate Gtf, tGtfA, adds *N*-acetylglucosamine (GlcNAc) to the β-OH of β-OH-Tyr_6, and finally, mannosylation occurs on residue 7 of the crosslinked heptapeptide by a mannosyltransferase. The teicoplanin family member A-40,926, also shown in Figure 1A, differs from teicoplanin by positions of aromatic ring chlorination, an N-terminal methyl group, the identity of the acyl chain, the absence of a GlcNAc at the β-OH of scaffold residue 6, and a C_6-COOH oxidation state on the *N*-decanoyl-sugar residue [5].

Perhaps the critical difference between teicoplanin, a lipoglycopeptide, and vancomycin, a glycopeptide, is the presence of the acyl chain which has been implicated in its mechanism of antimicrobial activity [6]. Teicoplanin retains efficacy against type B strains of vancomycin-resistant enterococci (VRE), while type A strains of VRE are resistant to both teicoplanin and vancomycin [7, 8]. The DNA sequences of biosynthetic gene clusters for the vancomycin analog chloroeremomycin [9], for teicoplanin [1, 2], and for the teicoplanin analogs A-49,734 [10] and A-40,926 [5] have been reported. This has enabled the identification and characterization of tailoring Gtfs [3, 4] and more recently of the tailoring acyltransferase (Atf) that adds the decanoyl group to the glucosamine moiety of teicoplanin [1].

The A-40,926 cluster, but not the A-49,734 cluster, has a homologous Atf [1, 5, 10], and we have now subcloned that enzyme and purified it from *E. coli*. The two enzymes share 67% sequence identity and 79% homology. We report the characterization and substrate specificities of both the teicoplanin Atf (tAtf) and the A-40,926 Atf (aAtf) using several glycopeptide intermediates from both the vancomycin and teicoplanin subfamilies as scaffold substrates and a variety of acyl-CoAs as acyl donor substrates. Remarkably, while the normal substrate for the Atfs is a 2-aminoglucosyl-heptapeptide scaffold, we find that a regioisomeric 6-aminoglucosyl substrate on both the teicoplanin scaffold and the vancomycin scaffold can be acylated at high pH. k_{cat} values of 0.2 min^{-1}, down about three orders of magnitude from 400–2000 min^{-1} determined here with natural 2-aminoglucosyl scaffolds, still suffice for preparative reactions. The N_6-acylglucosaminyl scaffolds can be further glycosylated, on the 2-OH, by the L-vancosaminyl transferase in the vancomycin maturation pathway to create new lipoglycopeptide antibiotics. This approach offers a chemoenzymatic route to this class of compounds which shows good activity against vancomycin-resistant strains [11–13].

Results

Cloning, Expression, and Purification of Acyltransferases

Spencer and colleagues have recently identified the acyltransferase in the teicoplanin biosynthetic gene cluster, *tei orf 11** (tAtf), and validated its activity after purification from *E. coli* overproduction, although no kinetic or specificity characterization was reported [1]. They have also proposed that *dbv orf 8* (aAtf) is the corresponding aminosugar *N*-acyltransferase from the A-40,926 producer [1]. In this paper we focus on the kinetic characterization and substrate specificities of these two enzymes. The two enzymes were PCR am-

*Correspondence: christopher_walsh@hms.harvard.edu

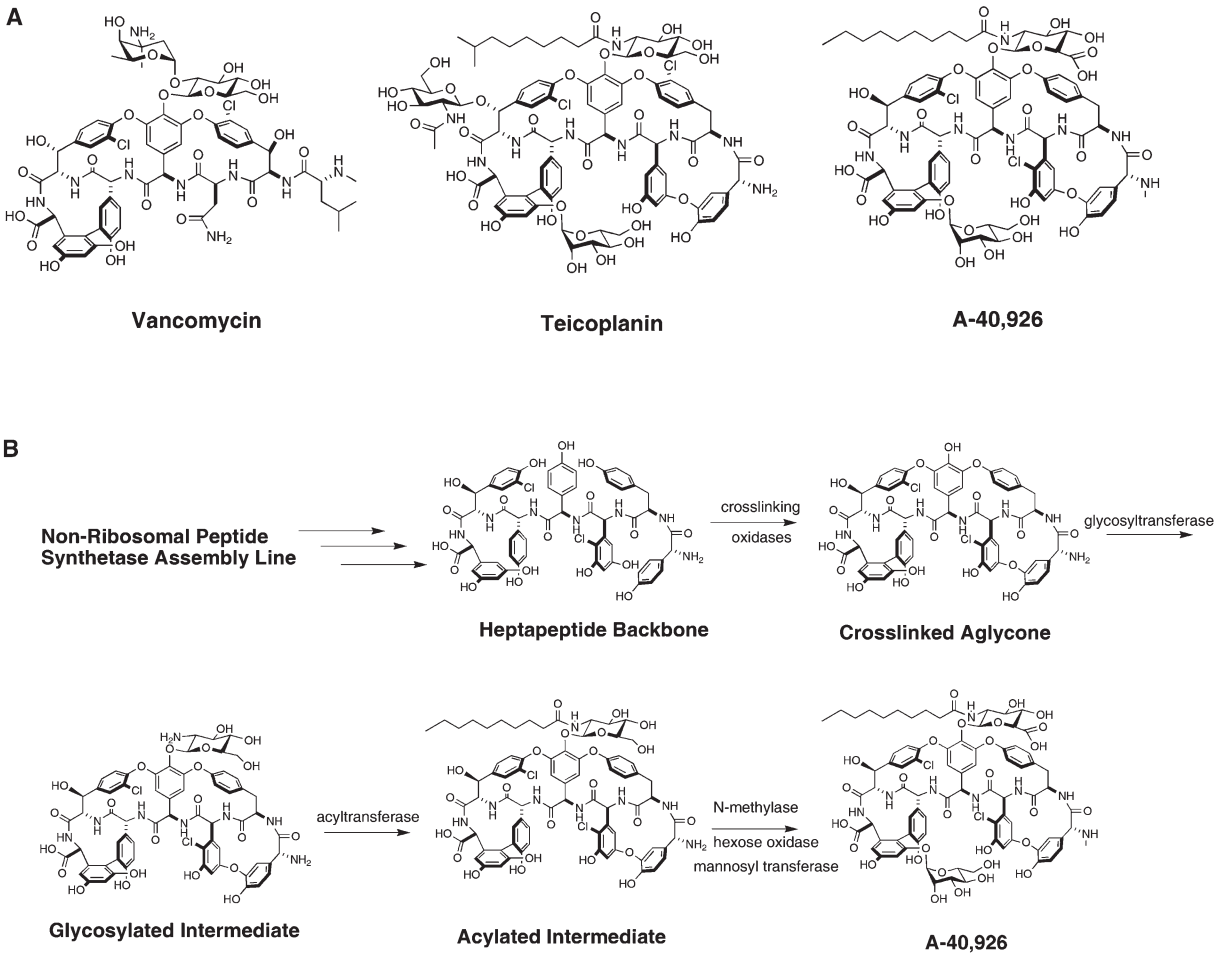

Figure 1. Biosynthesis of Glycopeptide Antibiotics

(A) Chemical structures of three important glycopeptide and lipoglycopeptide antibiotics.

(B) The linear peptide backbone of the glycopeptide family of antibiotics is synthesized by NRPS machinery. The backbone is then acted on by a number of tailoring enzymes including crosslinking oxidases, glycosyltransferases, acyltransferases, and methylases.

plified from cosmid DNA provided to us by the Donadio group at Vicuron Pharmaceuticals [2, 5], cloned into the pET22b expression vector, and expressed in *E. coli* as C-terminal His_6-tagged proteins. The proteins were purified by nickel chromatography to greater than 95% purity based on SDS-PAGE analysis with a yield of 27 mg protein per liter of cell culture for each enzyme (data not shown).

Chemoenzymatic Synthesis of Glycopeptide Scaffolds

The Atf enzymes acylate the sugar amino group of their corresponding 2-aminoglucosyl crosslinked heptapeptide scaffolds [1]. While there are slight variations between the teicoplanin and A-40,926 backbones (i.e., N-terminal methylation, differential aromatic chlorination, the absence of a GlcNAc at the β-OH of scaffold residue 6, and a C_6-COOH oxidation state on the N-decanoyl-sugar residue), we have chosen to use the teicoplanin scaffold due to its availability [5]. The teicoplanin aglycone (AGT) and vancomycin aglycone (AGV) can be obtained from teicoplanin or vancomycin through the acid hydrolysis of the sugar groups in trifluoroacetic acid [14, 15]. The aglycone can then be glycosylated with a variety of different sugar groups using the vancomycin or chloroeremomycin glycosyltransferases as previously described [3, 4]. We enzymatically synthesized 10 mg of 2-aminoglucosyl AGT, 6-aminoglucosyl AGT, glucosyl AGT, and 2-aminoglucosyl AGV, using the GtfE enzyme, the corresponding UDP-sugar, and the heptapeptide aglycone scaffold as shown in Figure 2A.

Since GtfE has relaxed aglycone specificity [4], we were also able to glycosylate scaffolds where the amino terminus was protected with the Alloc protecting group [6]. This allowed the synthesis of the N-decanoyl-6-aminoglucosyl AGT standard chemoenzymatically by enzymatically glycosylating the protected aglycone, chemically acylating the free amine on the 6-aminoglucosyl residue, and finally deprotecting the N terminus of the heptapeptide.

Initial Kinetic Characterization of the A-40,926 Acyltransferase

aAtf was incubated with 2-aminoglucosyl AGT and octanoyl-CoA, and the ensuing reaction was monitored by HPLC. A representative A_{280} HPLC chromatogram is

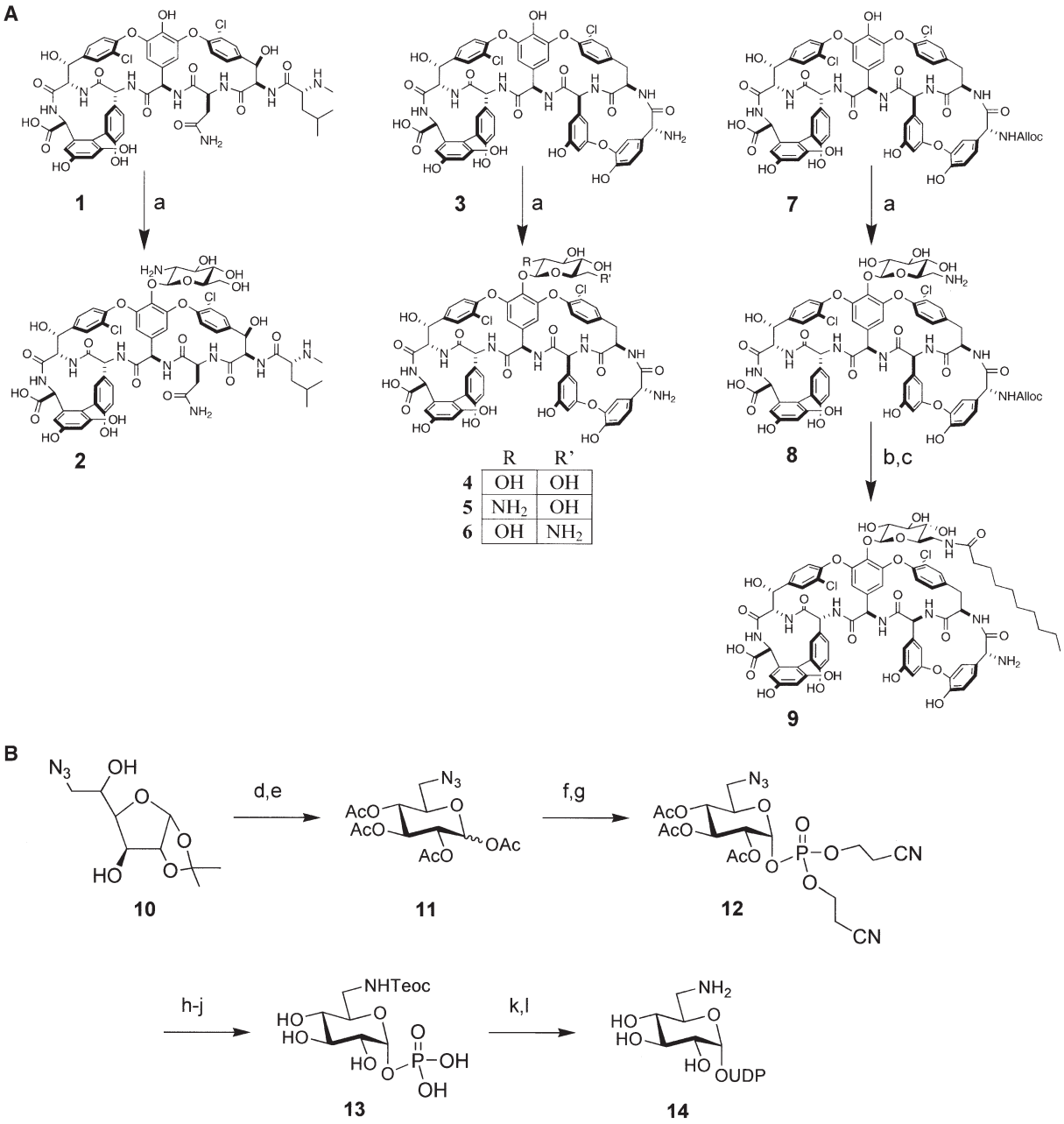

Figure 2. Synthesis of Glycopeptides and UDP-6-Aminoglucose

(A) Chemoenzymatic synthesis of glycopeptides used in this study. (a) 500 μM aglycone scaffold, 500 μM UDP-sugar, 450 μM GtfE. (b) decanoyl succinimide (3 equiv.), Et_3N, DMF, 16 hr, 57%. (c) $Me_2NH{\cdot}BH_3$ (6 equiv), $Pd(PPh_3)_4$ (0.4 equiv), DMF, 30 min, 66%.

(B) Synthesis of UDP-6-aminoglucose used in the chemoenzymatic synthesis of 6-aminoglucosyl-containing scaffolds. (d) $AcOH/H_2O$ 4:1; (e) Ac_2O, pyridine, DMAP, 81% (two steps); (f) hydrazine acetate, DMF, 73%; (g) 1. dicyanoethyl diisopropylphosphoramidite, tetrazole, CH_2Cl_2; 2. *m*CPBA, CH_2Cl_2; 3. isomerization with $BF_3{\cdot}OEt_2$, 69%; (h) NaOMe, MeOH; (i) H_2, Pd-C, MeOH; (j) Teoc succinimide, 80% (three steps); (k) UMP morpholidate, tetrazole, pyridine, 2 d; (l) Bu_4NF, DMF/THF, 45% (two steps).

shown in Figure 4E (bottom trace). The formation of a new product with a retention time of 12 min was observed, and the mass of the new peak determined by LCMS ($[M + H]^+$ = 1485.7) was consistent with the calculated mass of *N*-octanoyl-2-aminoglucosyl AGT ($[M + H]^+$ = 1485.4). To further verify the product of the acylation reaction, we compared a reaction mixture containing decanoyl-CoA to a previously reported authentic standard, *N*-decanoyl-2-aminoglucosyl AGT [6]. Coinjection of the reaction mixture and the standard showed that the enzymatic product coeluted with the chemically synthesized standard (data not shown). Steady-state kinetic analysis shows that the k_{cat} of aAtf is 480 min^{-1}, the K_m of 2-aminoglucosyl AGT is 136 μM, and the K_m of octanoyl-CoA is 22 μM at pH 7 (see Table 1). Parallel determination of the catalytic rate of tAtf re-

Table 1. aAtf Kinetic Parameters

Scaffold	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)	pH
2-NH_2-glucosyl AGT	480	136	3.53	7
2-NH_2-glucosyl AGV	214	209	1.02	7
2-NH_2-glucosyl AGT	592	18	32.9	9
6-NH_2-glucosyl AGT	0.253	29	0.0087	9
Glucosyl AGT	0.574	42	0.0137	9

Parameters reported are the average of triplicate experiments.

vealed a robust k_{cat} of 1800 min^{-1}, approximately 3-fold faster than the A-40,926 enzyme.

Acyl Chain Specificity

Teicoplanin and A-40,926 are naturally found as a mixture of related compounds differing in the identities of their acyl chains. The predominant form of A-40,926 has an *N*-decanoyl modification, while there are five major teicoplanin counterparts, all having between 10 and 11 carbons on their acyl chains [1, 2, 5]. These natural substrate tolerances led us to further explore the capabilities of these Atfs to act as combinatorial chemoenzymatic tools to generate libraries of novel lipoglycopeptides. The specificities of both Atfs were tested using the series of acyl-CoA substrates shown in Figure 3A. With the exception of hexynoyl-CoA and biphenoyl-CoA, which were synthesized using the general fatty acid/coenzyme A condensation strategy previously reported [16], all of these substrates were commercially available.

The results of the acyl chain specificity analysis are shown in Figures 3B and 3C. Both enzymes had a preference for long-chain acyl-CoAs. While trace amounts of product formation were observed using isobutyryl-, β-hydroxybutyryl-, crotonoyl-, and benzoyl-CoA by LCMS, the turnover was too slow to be quantified by HPLC. Biphenoyl-CoA was not a substrate for either enzyme. The best substrate for aAtf was the C_{12} acyl chain of lauroyl-CoA, while the best substrate for tAtf was decanoyl-CoA. In general the most robust substrates were acyl chains containing between 6 and 14 carbons. Across the acyl chain range, tAtf maintained a higher activity than aAtf.

Scaffold Specificity

The vancomycin and chloroeremomycin glycosyltransferases will accept either a vancomycin or a teicoplanin scaffold, allowing the chemoenzymatic synthesis of novel glycopeptides with modified sugar substituents [3, 4]. To ascertain whether the acyltransferases, which act on teicoplanin-type scaffolds, might share a similar scaffold tolerance, the ability of the Atfs to acylate 2-aminoglucosyl AGV was evaluated. Indeed, both aAtf and tAtf accept vancomycin scaffolds. Kinetic characterization of aAtf with 2-aminoglucosyl AGV and comparison to 2-aminoglucosyl AGT shows that both acceptor substrates are accepted nearly equally (Table 1). The k_{cat} for the vancomycin scaffold (214 min^{-1}) is 2-fold lower than that of the teicoplanin scaffold, and the K_m (209 μM) is about 2-fold higher, resulting in a 4-fold drop in catalytic efficiency. Although the complete kinetic characterization of the tAtf enzyme with 2-aminoglucosyl AGV was not performed, we qualitatively observed that tAtf could accept 2-aminoglucosyl AGV as a substrate as well (data not shown). The ability of these acyltransferases to acylate different scaffolds opens the door to generating novel lipoglycopeptides based on the vancomycin scaffold structure.

Sugar Specificity

In addition to scaffold and acyl chain specificities, the ability of the acyltransferase enzymes to recognize alternate sugars was examined. In the physiologic setting, the Atf enzymes recognize the nucleophilic amino nitrogen at the 2 position of the glucosyl ring. To see if acylation would proceed at other positions on the ring, 6-aminoglucosyl AGT was synthesized and tested. When

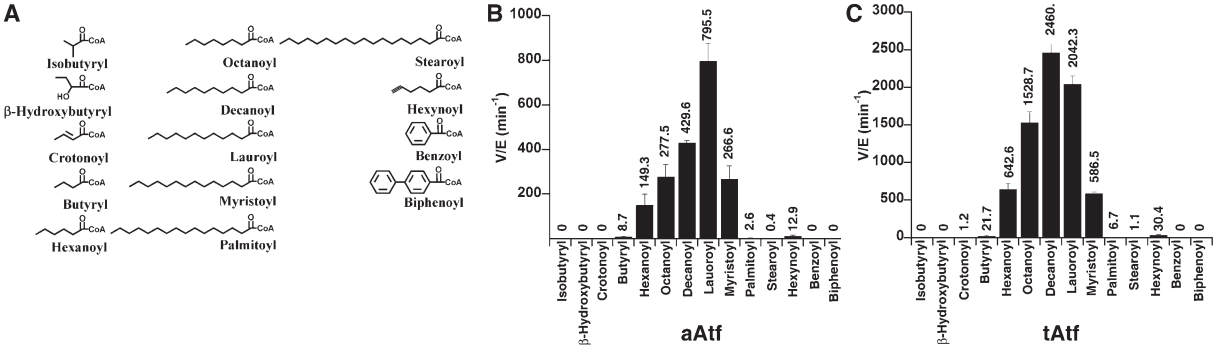

Figure 3. Acyl-CoA Specificity of aAtf and tAtf

(A) Chemical structures of acyl-CoAs used in this study.

(B and C) V/E comparison of the utilization of various acyl-CoA substrates using the aAtf and tAtf enzymes. The assay conditions are described in the Experimental Procedures.

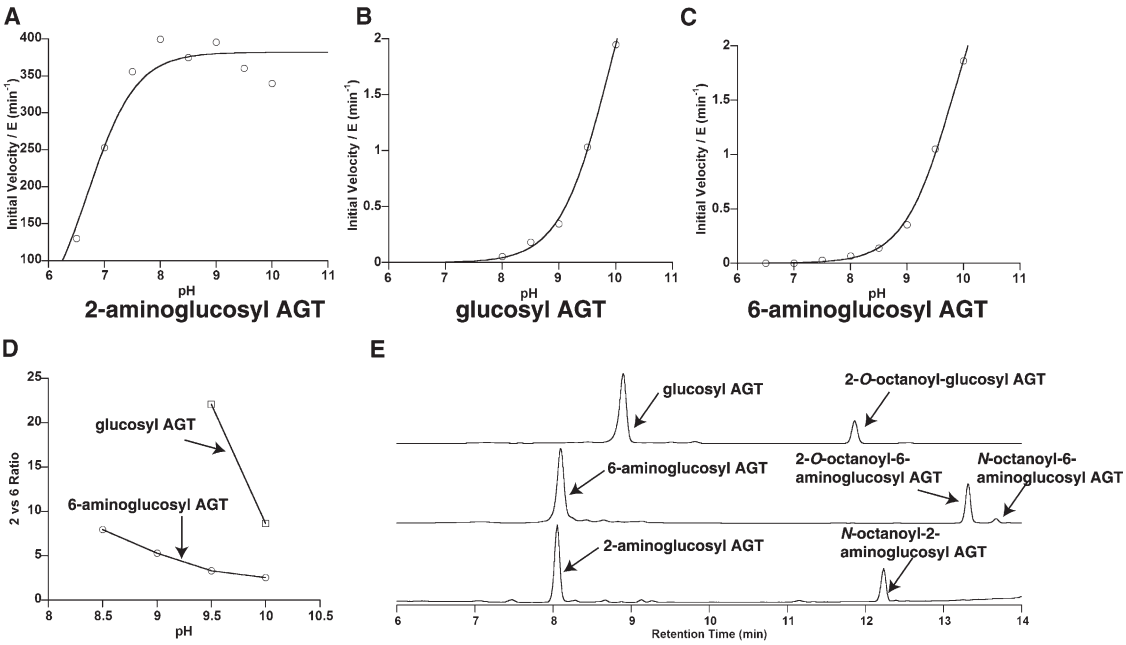

Figure 4. pH Dependence on aAtf Activity with Alternate Substrates

(A) pH dependence on 2-aminoglucosyl AGT utilization. Data were fit to a single ionization resulting in an estimated pKa of 6.7.

(B) pH dependence on glucosyl AGT utilization.

(C) pH dependence on 6-aminoglucosyl AGT utilization.

(D) Plot depicting how the ratio of products changes with increasing pH for reactions containing glucosyl AGT or 6-aminoglucosyl AGT.

(E) Representative A_{280} HPLC traces showing the substrates and products from reactions where 2-aminoglucosyl-, 6-aminoglucosyl-, and glucosyl AGT served as substrates at pH 7.0. The y axis is linear and is in units of absorbance.

this regioisomeric aminohexosyl scaffold was incubated with either Atf, two products could be detected (Figure 4E, middle trace), with one of the products in excess over the other.

LCMS analysis, as summarized in Table 2, provides insight into the identities of the two products. While the mass of each product is consistent with a single acylation of the scaffold, the major product flies as a doubly charged ion while the minor product flies only as a singly charged ion. Teicoplanin aglycone flies only as the singly charged ion (presumably ionization of the N-terminal amine). Both 2-aminoglucosyl- and 6-aminoglucosyl AGT fly as doubly charged ions (presumably both the N-terminal amine and the sugar amine are ionized). *N*-octanoyl-2-aminoglucosyl AGT flies only as the singly charged ion (the acylated sugar amine can no longer be ionized, and only the N-terminal amine remains ionizable). Since the major product of the acylation with 6-aminoglucosyl AGT flies as a doubly charged ion, we can deduce that the sugar amine remains unmodified, and acylation must have occurred at an alternate site, most likely the 2-OH position of the sugar, given the natural regiospecificity of the acyltransferase for the C_2 substituent of the hexose. Since the minor product flies as the singly charged ion, we presume that the sugar amine has been acylated and this is the N_6 octanoyl product, a novel site of acylation.

A chemically synthesized *N*-decanoyl-6-aminoglucosyl AGT standard was used for HPLC and LCMS comparison to the reaction products resulting from an incubation of aAtf with 6-aminoglucosyl AGT and decanoyl-CoA. HPLC coinjection verified that the chemically synthesized standard coeluted with the minor reaction product, and LCMS of the synthetic standard shows the singly charged ion (data not shown).

The ability of the acyltransferases to acylate sugar hydroxyls was confirmed by changing the sugar identity to glucose, thereby removing the amine altogether. Thus, glucosyl AGT could be utilized as an acylation substrate, and the formation of a new product could be detected by HPLC (Figure 4E, top trace). This new product, presumably the 2-O-decanoyl-glucosyl AGT,

Table 2. LCMS Results for Heptapeptide Acylation with Octanoyl-CoA

Scaffold	Theor.	Exp.	z	Theor. Product	Exp. Major Product	z	Exp. Minor Product	z
2-NH_2-glucosyl AGT	1359.3	1359.8	+2	1485.4	1485.7	+1	—	—
2-NH_2-glusocyl AGV	1304.4	1305.6	+2	1430.5	1431.0	+1	—	—
6-NH_2-glucosyl AGT	1359.3	1359.6	+2	1485.4	1486.6	+2	1486.2	+1
Glucosyl AGT	1360.3	1361.0	+1	1486.4	1487.1	+1	—	—

flies as a singly charged ion by LCMS analysis as expected, since there is only one amine available on the molecule for ionization. In order to provide NMR evidence for the position of acylation on glucosyl scaffolds, we choose to acylate the glucosyl vancomycin scaffold. We isolated 5 mg of the major acylated product by HPLC. The presence of a 2-*O*-acyl group was confirmed by its ^{1}H NMR spectrum (500 MHz, d_6-DMSO). The signal for the H-2 proton (δ = 4.85) of the glucose residue (assigned based on the COSY cross peaks) showed a distinct shift compared to the H-2 signal obtained for glucosyl vancomycin (δ = 3.55), characteristic for an acyl group attached to this position. No shift was observed at the other positions of the glucose ring.

To further explore this 2-OH and 6-NH_2 activity, we determined the pH optimum of aAtf using 2-aminoglucosyl-, 6-aminoglucosyl-, and glucosyl AGT. With the natural substrate (2-aminoglucosyl AGT), the pH dependence leveled off at pH 8 (Figure 4A). Fitting the pH dependence to a single ionization resulted in a pKa of 6.7. The pH dependence of both the glucosyl- and 6-aminoglucosyl AGT scaffolds shows an increasing rate with pH up to the pH of enzyme stability, pH 10 (Figures 4B and 4C). Interestingly, at higher pH values, a second minor monoacylated product could be seen forming in the glucosyl AGT reactions similar to the second minor product observed in the 6-aminoglucosyl AGT reactions. Due to the presence of two products, the velocity determined in these pH dependence experiments was taken as the sum of the rates of formation of each individual product. Figure 4D shows how the ratio between the two products changes as a function of pH for both substrates; at lower pH's acylation at C_2 predominates, while the amount of C_6 acylation increases at higher pH's.

While the activity was highest at pH 10, the formation of the minor product could be minimized by reducing the pH, and kinetic parameters for these substrates were determined at pH 9 (summarized in Table 1). The K_m for 2-aminoglucosyl AGT was reduced about one order of magnitude to 18 μM at pH 9 as compared to pH 7, resulting in a higher catalytic efficiency. The 6-aminoglucosyl- (29 μM) and glucosyl (42 μM) AGT scaffolds had similar K_m values as the natural substrate, while their k_{cat} values were reduced about 1000-fold (0.253 min^{-1} and 0.574 min^{-1}, respectively).

These results highlight two important findings: first, that tAtf and aAtf can catalyze the acylation of sugar hydroxyl groups, and second, that they can also acylate amines at position C_6 of the glucosyl ring. Although both activities occur at significantly reduced rates, they can be preparatively useful in the chemoenzymatic synthesis of novel lipoglycopeptides.

Synthesis of Novel Lipoglycopeptides

To demonstrate the utility of the glycosyltransferase and acyltransferase enzymes from the glycopeptide biosynthetic machinery to generate novel lipoglycopeptides, two lipoglycopeptide derivatives were generated as summarized in Figure 5. The first, compound **16**, is a teicoplanin analog that contains an acylated vancomycin disaccharide chain at $Phegly_4$ and is missing its *N*-acetylglucosamine sugar at β-OH-Tyr_6. This utilized the 6-aminoglucosyl AGT scaffold with aAtf-catalyzed enzymatic acylation of the 6-amino group on the sugar with decanoyl-CoA. Then, GtfD formed the *N*-acylated disaccharide by adding L-vancosamine (Figure 5A). In the second derivative, compound **19**, the *N*-acetylglucosamine sugar was added to the β-OH-Tyr_6 hydroxyl of 6-aminoglucosyl AGT, using tGtfA (the GtfA analog from the teicoplanin cluster). Then aAtf- and GtfD-mediated steps were employed sequentially to complete the three tailoring enzyme sequence (Figure 5A). All reactions were monitored by HPLC and by LCMS. Representative HPLC traces showing the formation of compound **16** are shown in Figure 5B.

Discussion

Natural products generated on nonribosomal peptide synthetase multimodular assembly lines commonly undergo a series of post assembly line tailoring reactions catalyzed by dedicated tailoring enzymes encoded in the biosynthetic genes clusters [17]. These enzymatic maturation steps may be crucial for the gain of biological activity, as typified by glycosylations of deoxyerythronolide B on the way to the antibiotic erythromycin and by the tandem oxidative crosslinkings of the acyclic heptapeptide to generate the rigid aglycone scaffolds for vancomycin and teicoplanin family antibiotics.

In toto, vancomycin undergoes three types of post assembly line enzymatic tailorings: Leu_1-*N*-methylation, the oxidative crosslinking of side chains 2–4, 4–6, and 5–7, and two glycosylations. Teicoplanin undergoes four oxidative crosslinkings, including side chains of residues 1–3, three glycosylations, and one acylation of the 2-aminoglucosyl moiety. The teicoplanin family member A-40,926 undergoes all of the teicoplanin modifications, except β-OH-Tyr_6 glycosylation, plus two additional ones: Leu_1-*N*-methylation and 2-aminoglucose double oxidation at C_6 to the COOH oxidation state (Figure 1).

In this study we have focused on the acyltransferases from the teicoplanin and A-40,926 clusters, the enzymes responsible for conversion of glycopeptide to lipoglycopeptide scaffolds. Sequencing of the clusters identified likely candidates for the Atfs [1, 5], and the activity of tAtf was validated experimentally by the studies of Spencer and colleagues that showed that, when heterologously expressed in and purified from *E. coli*, tAtf was active for acylation of the 2-aminoglucosyl moiety both on the crosslinked heptapeptide scaffold and as UDP-2-aminoglucose [1]. Here we have performed initial kinetic characterization of both the teicoplanin tAtf and the homologous A-40,926 aAtf for both scaffold and acyl-CoA substrates.

Both Atfs are robust catalysts, with k_{cat} values of 400 min^{-1} and 2000 min^{-1} for aAtf and tAtf, respectively, using the substrate pair 2-aminoglucosyl AGT and octanoyl-CoA at pH 7. Both Atfs show reasonable utilization of C_6-C_{14} acyl-CoA substrates but very low activities with short chain and aromatic acyl-CoAs.

Analysis of glycosyl-crosslinked heptapeptide scaffolds as substrates confirms that the 2-aminoglucosyl

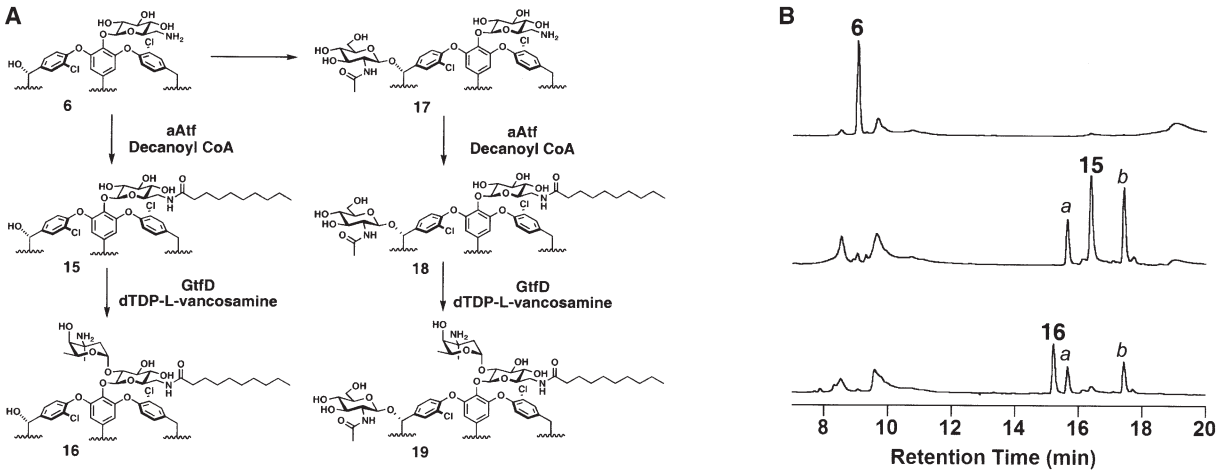

Figure 5. Chemoenzymatic Synthesis of Novel Lipoglycopeptides

(A) Schematic showing the synthetic strategy toward compounds **16** and **19** starting from 6-aminoglucosyl AGT.

(B) A_{280} HPLC traces showing the successive conversion of **6** to **15** and of **15** to **16** using aAtf and GtfD, respectively. The peak labeled "*a*" corresponds to 2-O-decanoyl-6-aminoglucosyl teicoplanin, while the peak labeled "*b*" corresponds to a doubly acylated product. Reaction conditions are described in the Experimental Procedures.

moiety is the preferred nucleophilic cosubstrate. As the pH is raised, detectable activity in the HPLC/MS assay is observed with glucosyl AGT for monoacylation with octanoyl-CoA as donor. Assuming the regiochemistry is maintained, this would represent a substitution of the 2-NH_2 group by the 2-OH group of the sugar. We isolated 5 mg of acylated glucosyl vancomycin scaffold, and NMR analysis shows a distinctive shift of the H-2 proton of the glucose ring confirming the site of acylation. The pKa of the -OH versus -NH_3^+ is about 5 units higher for generation of the 2-O^-, consistent with the monotonic rise of k_{cat} for the glucosyl acceptor substrate, while the 2-NH_2-substrate shows a plateau above pH 8.

When 6-aminoglucosyl AGT was evaluated as a substrate, a pH profile and k_{cat} comparable to the glucosyl-scaffold was observed. In this case two monoacylated products are detected (Figure 4E, middle trace). One of these is likely to be the 2-*O*-octanoyl-6-NH_2-glucosyl product, which appears to be the major kinetic product, while the other is likely to be the N_6-octanoyl-glucosyl product. We have verified the location of this N_6 acylation by comparing an authentic standard made chemically to the enzymatic product by HPLC and LCMS analysis. This apparent relaxation of the regioselectivity of the Atfs might be useful in the chemoenzymatic synthesis of glycopeptide derivatives where acyl chains can be appended to either the 2 or the 6 position of the glucosyl ring.

In particular, if the 2-OH of the 6-aminoglucosyl moiety is still free, one might expect this should enable further modification of the glucosyl moiety by vancosamine or epivancosamine by GtfD or GtfC, respectively. To that end the tandem incubations depicted in Figure 5 were conducted where the 6-aminoglucosyl AGT scaffold was subjected to Atf action, followed by GtfD and TDP-L-vancosamine. These were done in parallel following glycosylation of the β-OH of β-OH-Tyr_6 with GlcNAc by the teicoplanin tGtfA glycosyltransferase. In both cases decanoyl transfer was followed by vancosaminyl transfer to generate compounds **16** and **19**, containing the N_6-decanoyl-glucosyl-2-L-vancosaminyl substituent. These are lipoglycopeptides with the vancomycin-type disaccharide grafted on the teicoplanin scaffold, and the decanoyl side chain placed, not at N_2, but at N_6. Compound **16** is an analog of a vancomycin derivative previously synthesized chemically [13], while **19** has the three-sugar (disaccharide at residue four, monosaccharide at residue six) pattern characteristic of chloroeremomycin. The decanoyl-N_6 regiochemistry is intriguing and distinct from the *N*-chlorobiphenyl substitution on the amino group of the vancosaminyl sugar found in the clinical candidate oritavancin that is highly active against vancomycin-resistant enterococci.

Lipoglycopeptides are of interest, given that many of these compounds show improved activity against vancomycin-resistant bacteria [6, 11–13] and that two of the second-generation glycopeptides in clinical development, dalbavancin and oritavancin [18, 19], contain *N*-decanoyl and *N*-aryl substituents, respectively. The ability to decorate teicoplanin and vancomycin aglycone scaffolds with combinations of acyl-CoA and NDP-L- and NDP-D-hexoses (deoxy- and amino-) via action of Atfs and Gtfs should provide routes to new variants in the lipoglycopeptide class.

Significance

The vancomycin and teicoplanin group of natural product antibiotics share related crosslinked heptapeptide scaffolds that are produced by nonribosomal peptide synthetases and then become glycosylated by dedicated tailoring enzymes. One distinction between teicoplanin and vancomycin is that teicoplanin is a lipoglycopeptide, bearing a long-chain acyl group on the 2-aminoglucosyl moiety. We have now charac-

terized the recently discovered acyltransferase (Atf) from the teicoplanin biosynthetic cluster and a homologous Atf from the A-40,926 cluster with regard to specificity for acyl-CoA donors and glucosyl-heptapeptide nucleophilic acceptors. The ability of the Atfs to use both vancomycin and teicoplanin scaffolds, with glucosyl, 2-aminoglucosyl, and 6-aminoglucosyl acceptors, defines specificity parameters for creation of novel lipoglycopeptide variants. In particular, the ability of the Atfs to make N_6-long-chain acyl derivatives of 6-aminoglucosyl scaffolds, followed by regiospecific Gtfs, demonstrates that tandem Atf and Gtf action may allow enzymatic decoration to create novel lipoglycopeptide antibiotics.

Experimental Procedures

General

All chemicals were purchased from Aldrich or Sigma and used without further purification. Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on aluminum plates precoated with silica gel (250 μm, Sigma), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32–63 μm) purchased from Sorbent Technologies. NMR spectra were recorded on Varian Mercury 300 MHz and Inova 400 and 500 MHz spectrometers. Mass spectra (ESI) were obtained at the Mass Spectroscopy Facility at the Department of Chemistry, Princeton University, or with a Shimadzu QP8000 LCMS.

aAtf and tAtf Plasmid Construction

The genes responsible for encoding aAtf and tAtf, *dbv orf 8* and *tei orf 11**, respectively, were PCR amplified from cosmids provided by the Donadio group at Vicuron Pharmaceuticals [2, 5] using the following primer pairs: 5′-GGAATTCCATATGGATGCTGAGAGCGTG CGCAGGCAGC-3′ and 5′-CCCAAGCTTGGGCAGCTCGATGCGGC CGCTCC-3′ for *dbv orf 8* and 5′-GGAATTCCATATGGATCCCGA GACCGTACGCATCG-3′ and 5′-CCGCTCGAGCGGCAGCTTGAAG CCCGTCCGCAT-3′ for *tei orf 11**, where the restriction sites for NdeI, HindIII, and XhoI are underlined. The PCR products were cloned into pET22b expression vectors generating the pET-22b-aAtf-His_6 and pET-22b-tAtf-His_6 plasmids, and the open reading frames were confirmed by DNA sequencing prior to transformation into *E. coli* BL21DE3.

Heterologous Expression and Purification of aAtf and tAtf

E. coli BL21DE3 transformants were inoculated into 10 ml of LB medium containing 100 μg/ml ampicillin and were grown at 37°C overnight. Each overnight culture was added to 1 liter of LB containing 100 μg/ml ampicillin, and the cultures were incubated at 37°C until an OD_{600} of 0.6 was reached. The cultures were cooled to 15°C and were induced with 0.3 mM IPTG for 20 hr. Bacteria were harvested by centrifugation, and the cell pellets were resuspended in buffer A (25 mM MOPS [pH 7.0], 100 mM NaCl, and 5 mM imidazole). Bacteria were lysed in a French press (3 × 15,000 psi), and cell debris was removed by centrifugation (14,000 × g, 20 min). Cell lysates were mixed with 1 ml of preequilibrated Ni-NTA resin at 4°C for 1 hr before loading into a column. The column was washed with 10 column volumes of buffer A followed by 10 column volumes of buffer A containing 30 mM imidazole. The proteins were eluted with buffer A containing 200 mM imidazole, and fractions were analyzed by 12% SDS-PAGE. The desired fractions were pooled, transferred to a Slide-A-Lyzer dialysis cassette (10 kDa MW cutoff), and dialyzed twice against 2 liters of dialysis buffer (50 mM MOPS [pH 7.0], 100 mM NaCl, and 10% (v/v) glycerol). The protein concentrations were determined from their theoretical extinction coefficients at 280 nM (67,380 M^{-1} cm^{-1} for aAtf and 73,910 M^{-1} cm^{-1} for tAtf) [20, 21]. The proteins were flash frozen at 280 μM in liquid nitrogen and stored at −80°C.

Cloning, Expression, and Purification of *tGtfA*

The *tgtfA* gene was PCR amplified from the plasmid pAT9 using the following primer pair: 5′-AAAAAACATATGCGCGTGCTGTTTT CGTCCTAC-3′ and 5′-AAAAAAAAGCTTCGCGGGAACCGACGATCT-3′, in which the NdeI and HindIII sites are underlined. The PCR product was cloned into the pET22b expression vector (Novagen), and the open reading frame of *tgtfA* was confirmed by DNA sequencing before it was transformed into BL21(DE3)-competent cells (Invitrogen). The tGtfA protein was expressed and purified using a previously described method [22].

Chemoenzymatic Synthesis of Glycopeptide Scaffolds

Vancomycin aglycone (AGV) **1** and teicoplanin aglycone (AGT) **3** were synthesized as previously described [14, 15]. These aglycones were then enzymatically glycosylated [3] with UDP-glucose, UDP-2-aminoglucose, or UDP-6-aminoglucose using the vancomycin biosynthetic glycosyltransferase GtfE to yield the monoglycosylated scaffolds **2** and **4**–**6** as shown in Figure 2A. The reaction conditions were as follows: 75 mM Tricine (pH 9.0), 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/mL BSA, 10% (v/v) DMSO, 500 μM UDP-sugar, 500 μM aglycone, and 450 μM GtfE. The reactions were monitored by HPLC, and additional aliquots of GtfE were added twice daily until the reaction proceeded to 50% completion (up to 3 days). The resulting monoglycosylated scaffolds were purified by preparative HPLC (Vydac C_{18} column, 0%–30% acetonitrile, 0.1% TFA in 25 min) and were verified by LCMS. For the synthesis of **9**, the N terminus of **3** was Alloc protected to generate compound **7** as described previously [6]. The protected aglycone was then glycosylated with UDP-6-aminoglucose to generate glycopeptide **8**. Alloc-protected glycopeptide **8** was then acylated with a decanoyl chain and deprotected to generate **9** as described for the corresponding 2-NH-decanoyl analog previously synthesized [6]. The NDP-sugars used for the enzymatic glycosylations were obtained as follows: UDP-glucose was commercially available, UDP-2-aminoglucose [23] and TDP-L-vancosamine [24] were synthesized as previously described, and UDP-6-aminoglucose, **14**, was synthesized as described below (see Figure 2B).

1,2,3,4-Tetra-O-Acetyl-6-Azido-6-Deoxy-D-Glucopyranose (11)

A suspension of 6-azido-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**10**; 10.0 g, 40.7 mmol) [25] in AcOH/H_2O (4:1, 300 ml) was stirred at 80°C for 16 hr. The resulting solution was evaporated, coevaporated with toluene (3×), and the residue was dissolved in pyridine (100 ml). After the addition of Ac_2O (50 ml) and DMAP (500 mg), the solution was stirred at RT for 8 hr. Following evaporation, the residue was dissolved in CH_2Cl_2 and washed with 1 N HCl, satd. aq. $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), evaporated, and coevaporated with toluene, and the residue was crystallized from petroleum ether/AcOEt to give the tetraacetate **11** (12.3 g, 81%) as a 1:1 mixture of anomers. ^1H NMR ($CDCl_3$, 300 MHz): δ 6.34 (d, $J_{1,2}$ = 3.6 Hz, H-1α), 5.71 (d, $J_{1,2}$ = 8.2 Hz, H-1β), 5.45, 5.34, 5.15–5.03 (t, t, m, H-2, H-3, H-4), 4.12–4.02, 3.84–3.76 (2 m, H-5α, H-5β), 3.42–3.25 (m, 6-H_2), 2.17, 2.11, 2.05, 2.04, 2.02, 2.01 (OAc).

Dicyanoethyl (2,3,4-Tri-O-Acetyl-6-Azido-6-Deoxy-α-D-Glucopyranosyl) Phosphate (12)

A solution of tetraacetate **11** (6.0 g, 16.1 mmol) and hydrazine acetate (1.93 g, 20.9 mmol) in DMF (18 ml) was stirred at 65°C for 30 min. After cooling to RT, the solution was diluted with EtOAc (500 ml) and washed with H_2O (2 × 150 ml), satd. aq. $NaHCO_3$ (2 × 150 ml), and brine (150 ml), and the aqueous phases were back-extracted with EtOAc. The combined organic phases were dried ($MgSO_4$), evaporated, and purified by flash chromatography on silica gel (petroleum ether/EtOAc 2:1) to give the lactol as a colorless syrup (3.9 g, 73%). The lactol (3.3 g, 10 mmol) and tetrazole (2.8 g, 40 mmol) were coevaporated with toluene (3×), dissolved in CH_2Cl_2 (80 ml), and cooled to −5°C. A solution of dicyanoethyl diisopropylphosphoramidite (5.42 g, 20 mmol) in CH_2Cl_2 (80 ml) was then added, and the temperature was allowed to reach RT over the course of 1.5 hr. The solution was cooled to −40°C, a solution of *m*CPBA (3.5 g, 20 mmol) in CH_2Cl_2 (80 ml) was added, and the reaction mixture was warmed to RT over 1 hr. After the addition of

aq. Na_2SO_3 (10%, 100 ml), the mixture was extracted with CH_2Cl_2, and the organic phase was washed with satd. aq. $NaHCO_3$ (2×) and brine. The organic phase was dried ($MgSO_4$), evaporated, and purified by flash chromatography on silica gel (petroleum ether/EtOAc 1:9) to give the phosphate **12** (4.6 g; R_f = 0.1, petroleum ether/EtOAc 1:4) as a mixture of anomers (α/β = 3:1 according to ^{1}H NMR). Removal of the undesired β-anomer using silica gel chromatography was unsuccessful; therefore, the anomeric mixture was isomerized to the more stable α-anomer using a catalytic amount of $BF_3{\cdot}OEt_2$ in CH_2Cl_2. After 1.5 hr, the reaction was neutralized by adding a few drops of Et_3N, washed with brine, dried ($MgSO_4$), evaporated, and purified by flash chromatography on silica gel (petroleum ether/EtOAc 1:6, then pure EtOAc) to give the α-anomer of phosphate **12** (3.6 g, 69%) as a colorless syrup. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.92 (dd, 1 H, $J_{1,2}$ = 3.3, $J_{1,P}$ = 6.6 Hz, H-1), 5.46, 5.09 (2 t, each 1 H, J = 10.1 Hz, H-3, H-4), 5.03–4.98 (m, 1 H, H-2), 4.40–4.30 (m, 4 H, OCH_2), 4.22–4.16 (m, 1 H, 5-H), 3.45 (dd, 1 H, $J_{5,6a}$ = 2.2, $J_{6,6}$ = 13.5 Hz, H-6a), 3.37 (dd, 1 H, $J_{5,6b}$ = 5.9, $J_{6,6}$ = 13.5 Hz, H-6b), 2.88–2.80 (m, 4 H, CH_2CN), 2.09, 2.05, 2.02 (3 s, each 3 H, 3 OAc); MS (ESI) for $C_{18}H_{24}N_5O_{11}P$ (517.38): 516 $[M–H]^–$.

6-(2-Trimethylsilylethyloxycarbonyl)-Amino-6-Deoxy-α-D-Glucopyranosyl Phosphate (13)

To a solution of phosphate **12** (725 mg, 1.4 mmol) in MeOH (20 ml) was added NaOMe (7 ml, 0.5 M in MeOH, 3.5 mmol), and the mixture was stirred at 55°C for 30 min. After evaporation of the solvent, the residue was dissolved in H_2O (30 ml). The solution was neutralized with Amberlite IRC50, filtered, and extracted with Et_2O (20 ml). Pd-C (400 mg) was then added, and the suspension was stirred under H_2 (1 atm) for 3 hr, filtered, and evaporated to a final volume of ca. 10 ml. After the addition of Teoc succinimide (540 mg, 2.1 mmol) and Et_3N (0.5 ml), the mixture was stirred for 16 hr and evaporated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH/H_2O/Et_3N 16:4:0.5:0.3, then 16:8:2:0.5) to afford phosphate **13** (630 mg, 80%; R_f = 0.2, CH_2Cl_2/MeOH/H_2O/Et_3N 16:4:0.5:0.3) as the triethylammonium salt (1.6 eq.). ^{1}H NMR (CD_3OD, 400 MHz): δ 5.47 (dd, 1 H, $J_{1,2}$ = 3.5, $J_{1,P}$ = 6.9 Hz, H-1), 4.13–4.05 (m, 2 H, OCH_2), 3.85–3.78 (m, 1 H, H-5), 3.64 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.52 (d, 1 H, $J_{6,6}$ = 12.3 Hz, H-6a), 3.36–3.32 (m, 1 H, H-2), 3.22–3.12 (m, 2 H, H-4, H-6b), 1.27 (t, 2 H, CH_2 $SiMe_3$), 0.02 (s, 9 H, $SiMe_3$); ^{31}P NMR (D_2O, 162 MHz): δ 1.40; MS (ESI) for $C_{12}H_{26}NO_{10}PSi$ (403.4): 402 $[M–H]^–$.

Uridine 5′-(6-Amino-6-Deoxy-α-D-Glucopyranosyl Diphosphate) (14)

Phosphate **13** (590 mg, 1.05 mmol) and trioctylamine (0.47 ml, 1.05 mmol) were coevaporated with pyridine (3×). UMP morpholidate (1.15 g, 1.67 mmol) was added, and the mixture was coevaporated with pyridine (3×). The residue was dissolved in pyridine (8 ml), tetrazole (250 mg, 3.2 mmol) was added, and the mixture was stirred for 2 days. Following evaporation, the residue was dissolved in H_2O containing 0.1% NH_4HCO_3 (18 ml), extracted with Et_2O, and purified by preparative reversed-phase HPLC to give the protected UDP derivative (420 mg, 56%). This compound was dissolved in DMF (5 ml), Bu_4NF (1 M in THF, 5 ml) was added, and the solution was stirred for 16 hr. After removal of the solvents, the residue was dissolved in H_2O containing 0.1% NH_4HCO_3 (10 ml), filtered, and purified by preparative reversed-phase HPLC to afford the UDP sugar **14** (265 mg, 80%) as its ammonium salt. ^{1}H NMR (D_2O, 400 MHz): δ 7.98 (d, 1 H, J = 8.1 Hz, uridine Cnb-H), 5.99–5.95 (m, 2 H, uridine Cnb-H, H-1 [ribose]), 5.63 (dd, 1 H, $J_{1,2}$ = 3.5, $J_{1,P}$ = 6.9 Hz, Hnb-1), 4.40–4.20 (m, 5 H, H-2, H-3, H-4, H-5 [ribose]), 4.12 (dt, 1 H, $J_{4,5}$ = $J_{5,6b}$ = 9.9, $J_{5,6a}$ = 2.7 Hz, H-5), 3.78 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 3.60 (dt, 1 H, $J_{1,2}$ = $J_{2,P}$ = 3.5, $J_{2,3}$ = 9.8 Hz), 3.48 (dd, 1 H, $J_{5,6}$ = 2.7, $J_{6,6}$ = 13.2 Hz, H-6a), 3.36 (t, 1 H, $J_{3,4}$ = 9.8, $J_{4,5}$ = 9.9 Hz, H-4), 3.11 (dd, 1 H, $J_{5,6b}$ = 9.9, $J_{6,6}$ = 13.2 Hz, H-6b); ^{31}P NMR (D_2O, 162 MHz): δ –10.4, –12.1; MS (ESI) for $C_{15}H_{25}N_3O_{16}P_2$ (565.3): 564 $[M–H]^–$.

Initial Kinetic Characterization of aAtf

Octanoyl-CoA (25–200 μM) and either 2-aminoglucosyl AGT (70–700 μM) or 2-aminoglucosyl AGV (90–900 μM) were incubated at 37°C with 40 nM aAtf in 25 μl of reaction buffer (75 mM Tris [pH 7.0], 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/ml BSA, and 10% [v/v] DMSO) for 2 min, after which the reactions were quenched by the addition of 50 μl methanol. The quenched reactions were centrifuged for 2 min at 13,000 rpm, and 20 μl of the supernatant was injected into a Vydac analytic C_{18} HPLC column. Reaction mixtures were eluted using a gradient from 10% to 75% acetonitrile and 0.1% TFA in 20 min, and products were monitored at 280 nm. Negative control reactions were performed for all assays where enzyme was left out to verify there was no detectable nonenzymatic reaction. Molecular weights of substrates and products were verified by LCMS. The peaks for the acylated products and the unacylated substrates were integrated, and the product concentration was deduced from its percentage of the total peak area. The resulting initial velocity data were fit to the Michaelis-Menten equation to generate the kinetic parameters k_{cat} and K_m. Full kinetic characterization of tAtf was not performed, and instead an estimate of the k_{cat} for tAtf was determined by incubating 40 nM tAtf with saturating amounts of both substrates (700 μM 2-aminoglucosyl AGT and 500 μM octanoyl-CoA) using the assay conditions outlined above allowing direct comparison to aAtf.

Atf Acyl Chain Specificity

aAtf and tAtf were incubated with 100 μM of various acyl-CoA substrates (see Figure 3A) and 100 μM 2-aminoglucosyl AGT using the assay conditions outlined above. The enzyme concentration varied between 50 nM and 4 μM depending on the reactivity of each acyl-CoA substrate. Initial velocities were determined for each acyl-CoA with both Atfs in triplicate. The resulting velocity was normalized by the enzyme concentration to generate V/E, which was plotted for each acyl-CoA.

Atf Sugar Specificity

The kinetic parameters k_{cat} and K_m were determined for the teicoplanin scaffold containing one of three different sugars (2-aminoglucose, 6-aminoglucose, or glucose) at pH 9.0. aAtf was incubated with 50–2000 μM scaffold and 1 mM octanoyl-CoA at 37°C in 25 μl of reaction buffer (75 mM Tris [pH 9.0], 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/ml BSA, and 10% [v/v] DMSO). In the 2-aminoglucosyl AGT reactions, 40 nM aAtf was used with 2 min reaction times, while in the 6-aminoglucosyl AGT and glucosyl AGT reactions, 5 μM aAtf was used with 10 min reaction times. The reactions were quenched and HPLC analyzed as described above.

To identify the sites of acylation for these substrates, comparison of reaction products to known chemical standards or NMR analysis was performed. 2-*N*-decanoyl-glucosyl AGT and 6-*N*-decanoyl-glucosyl AGT were coinjected with the corresponding reaction mixtures to verify acylation at the C2 or C6 amine, respectively. Since an authentic standard for the acylation of a glucosyl scaffold was unavailable, 5 mg of the major reaction product between glucosyl vancomycin and decanoyl-CoA was purified by HPLC, and the ^{1}H NMR spectrum was determined (500 MHz, d_6-DMSO). The spectrum of the reaction product was compared to that of glucosyl vancomcyin showing a distinctive shift in the H-2 proton of the glucose residue while the other protons remained unaffected.

pH Dependence of aAtf Activity

The pH dependence of the aAtf reaction was determined using 2-aminoglucosyl-, 6-aminoglucosyl-, or glucosyl AGT as acceptor substrates over a pH range from 6.5 to 10 at every 0.5 pH unit. Reactions were performed with 200 μM scaffold, 500 μM octanoyl-CoA, at 37°C in 25 μl of reaction buffer (8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/ml BSA, and 10% [v/v] DMSO), which included 75 mM HEPES (pH 6.5–7.0), Tris (pH 7.0–9.0), or CHES (pH 9.0–10.0). In the 2-aminoglucosyl AGT reactions, 40 nM aAtf was used with 2 min reaction times, while in the 6-aminoglucosyl AGT and glucosyl AGT reactions, 5 μM aAtf was used with 10 min reaction times. The reactions were quenched and HPLC analyzed as described above.

Synthesis of Novel Lipoglycopeptides Using Gtfs and Atfs

To make compound **16** (see Figure 5A), 500 μM 6-aminoglucosyl AGT **6**, 1 mM decanoyl-CoA, and 1 μM tAtf were incubated in 100 μl of reaction buffer A (50 mM Tris [pH 9.0] and 1 mg/ml BSA) at 37°C for 60 min, generating compound **15**. Two byproducts were

formed, the 2-O-decanoyl-6-aminoglucosyl AGT and a doubly acylated product as shown in Figure 5B. 40 μl of the resulting mixture was adjusted to a pH of 7.0 and further incubated with 500 μM TDP-L-vancosamine and 2 μM GtfD at 37°C for 3 hr, resulting in compound **16**. To make compound **19**, 500 μM 6-aminoglucosyl AGT, 1 mM UDP-GlcNAc, and 5 μM tGtfA were incubated in 500 μl of reaction buffer B (50 mM Tris [pH 7.5] and 2.5 mM TCEP) at 37°C for 12 hr to generate compound **17**. Compound **17**, purified by RP-HPLC using a Vydac C_{18} small-pore column, was incubated with 1 mM decanoyl-CoA in the presence of 1 μM tAtf in 100 μl of reaction buffer A at 37°C for 60 min, generating compound **18**. Half of the resulting reaction mixture was incubated with 500 μM TDP-L-vancosamine and 2 μM GtfD at 37°C for 3 hr to produce compound **19**. All reaction mixtures were analyzed by both HPLC and LCMS, and when available, products were confirmed by coinjection with authentic chemically synthesized standards.

Acknowledgments

This work was supported by NIH grant GM49338 to C.T.W. and NIH grant GM66174 to D.K. J.T. thanks K. Albizati and V. Martin for generous support of secondment.

Received: August 30, 2004
Revised: October 22, 2004
Accepted: October 30, 2004
Published: January 21, 2005

References

1. Li, T.L., Fanglu, H., Haydock, S.F., Mironenko, T., Leadlay, P.F., and Spencer, J.B. (2004). Bioynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glycosyltransferases and the key acyltransferase. Chem. Biol. *11*, 107–119.
2. Sosio, M., Kloosterman, H., Bianchi, A., de Vreugd, P., Dijkhuizen, L., and Donadio, S. (2004). Organization of the teicoplanin gene cluster in *Actinoplanes teichomyceticus*. Microbiol. *150*, 95–102.
3. Losey, H.C., Peczuh, M.W., Chen, Z., Eggert, U.S., Dong, S.D., Pelczer, I., Kahne, D., and Walsh, C.T. (2001). Tandem action of glycosyltransferases in the maturation of vancomycin and teicoplanin aglycones: novel glycopeptides. Biochemistry *40*, 4745–4755.
4. Losey, H.C., Jiang, J., Biggins, J.B., Oberthur, M., Ye, X.Y., Dong, S.D., Kahne, D., Thorson, J.S., and Walsh, C.T. (2002). Incorporation of glucose analogs by GtfE and GtfD from the vancomycin biosynthetic pathway to generate variant glycopeptides. Chem. Biol. *9*, 1305–1314.
5. Sosio, M., Stinchi, S., Beltrametti, F., Lazzarini, A., and Donadio, S. (2003). The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *nonomuraea* species. Chem. Biol. *10*, 541–549.
6. Dong, S.D., Oberthur, M., Losey, H.C., Anderson, J.W., Eggert, U.S., Peczuh, M.W., Walsh, C.T., and Kahne, D. (2002). The structural basis for induction of VanB resistance. J. Am. Chem. Soc. *124*, 9064–9065.
7. Johnson, A.P., Uttley, A.H., Woodford, N., and George, R.C. (1990). Resistance to vancomycin and teicoplanin: an emerging clinical problem. Clin. Microbiol. Rev. *3*, 280–291.
8. Banerjee, S.N., Emori, T.G., Culver, D.H., Gaynes, R.P., Jarvis, W.R., Horan, T., Edwards, J.R., Tolson, J., Henderson, T., and Martone, W.J. (1991). Secular trends in nosocomial primary bloodstream infections in the United States, 1980–1989. National Nosocomial Infections Surveillance System. Am. J. Med. *91*, 86S–89S.
9. van Wageningen, A.M., Kirkpatrick, P.N., Williams, D.H., Harris, B.R., Kershaw, J.K., Lennard, N.J., Jones, M., Jones, S.J., and Solenberg, P.J. (1998). Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. Chem. Biol. *5*, 155–162.
10. Pootoolal, J., Thomas, M.G., Marshall, C.G., Neu, J.M., Hubbard, B.K., Walsh, C.T., and Wright, G.D. (2002). Assembling the glycopeptide antibiotic scaffold: the biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. Proc. Natl. Acad. Sci. USA *99*, 8962–8967.
11. Allen, N.E., and Nicas, T.I. (2003). Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol. Rev. *26*, 511–532.
12. Ge, M., Chen, Z., Onishi, H.R., Kohler, J., Silver, L.L., Kerns, R., Fukuzawa, S., Thompson, C., and Kahne, D. (1999). Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. Science *284*, 507–511.
13. Kerns, R., Dong, S.D., Fukuzawa, S., Carbeck, J., Kohler, J., Silver, L., and Kahne, D. (2000). The role of hydrophobic substituents in the biological activity of glycopeptide antibiotics. J. Am. Chem. Soc. *122*, 12608–12609.
14. Malabarba, A., Ferrari, P., Gallo, G.G., Kettenring, J., and Cavalleri, B. (1986). Teicoplanin, antibiotics from *Actinoplanes teichomyceticus* nov. sp. VII. Preparation and NMR characteristics of the aglycone of teicoplanin. J. Antibiot. (Tokyo) *39*, 1430–1442.
15. Marshall, F.J. (1965). Structure studies on vancomycin. J. Med. Chem. *39*, 18–22.
16. Belshaw, P.J., Walsh, C.T., and Stachelhaus, T. (1999). Aminoacyl-CoAs as probes of condensation domain selectivity in nonribosomal peptide synthesis. Science *284*, 486–489.
17. Walsh, C.T., Chen, H., Keating, T.A., Hubbard, B.K., Losey, H.C., Luo, L., Marshall, C.G., Miller, D.A., and Patel, H.M. (2001). Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. Curr. Opin. Chem. Biol. *5*, 525–534.
18. Malabarba, A., and Ciabatti, R. (2001). Glycopeptide derivatives. Curr. Med. Chem. *8*, 1759–1773.
19. Malabarba, A., Nicas, T.I., and Thompson, R.C. (1997). Structural modifications of glycopeptide antibiotics. Med. Res. Rev. *17*, 69–137.
20. Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. Protein Sci. *4*, 2411–2423.
21. Gill, S.C., and von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. *182*, 319–326.
22. Lu, W., Leimkuhler, C., Oberthur, M., Kahne, D., and Walsh, C.T. (2004). AknK is an L-2-deoxyfucosyltransferase in the biosynthesis of the anthracycline aclacinomycin A. Biochemistry *43*, 4548–4558.
23. Lu, W., Oberthur, M., Leimkuhler, C., Tao, J., Kahne, D., and Walsh, C.T. (2004). Characterization of a regiospecific epivancosaminyl transferase GtfA and enzymatic reconstitution of the antibiotic chloroeremomycin. Proc. Natl. Acad. Sci. USA *101*, 4390–4395.
24. Oberthur, M., Leimkuhler, C., and Kahne, D. (2004). A practical method for the stereoselective generation of β-2-deoxy glycosyl phosphates. Org. Lett. *6*, 2873–2876.
25. Fleet, G.W.J., Ramsden, N.G., and Witty, D.R. (1989). A practical synthesis of deoxymannojirimycin and of (2S,3R,4R,5R)-3,4,5-trihydroxypipecolic acid from D-glucose. Tetrahedron *45*, 327–336.